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Combination of pH-controlled fermentation in mild acidic conditions and enzymatic hydrolysis by Savinase to improve metabolic health-promoting properties of lentil

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ABSTRACT

This work evaluated the feasibility of pH-controlled fermentation by *Lactobacillus plantarum* CECT 748 combined with Savinase-hydrolysis (LPHS) for producing multifunctional lentil ingredients targeted for metabolic syndrome (MetS) relieving. LPHS process was compared with *L. plantarum*-fermentation (LP) and Savinase-hydrolysis (HS), applied separately. LPHS soluble fraction exhibited higher peptides, *p*-hydroxybenzoic acid, flavonols, antioxidant (400.74 mM TE/g), angiotensin I-converting enzyme (ACE) (95.43%) and α-glucosidase-inhibitory activities (40.55 and 25.03% for maltase and sacarase activities, respectively) than LP and HS. *L. plantarum* was responsible for the phenolic profile changes and sucrase inhibitory activity of LPHS while Savinase contributed to peptides release and ACE and maltase inhibitory and antioxidant activities. The most active LPHS fraction contained 3 peptides with potential biological activity and flavonoids and phenolic acids. LPHS simulated gastrointestinal digestion enhanced its peptides, phenolics, ACE-inhibitory and antioxidant activities. This study opens new opportunities regarding the production of lentil-multifunctional ingredients for MetS management.

1. Introduction

Chronic diseases are increasingly viewed as a global health concern due to their escalating incidence and because they are responsible for 70% of deaths worldwide. Metabolic risk factors as raised blood pressure, overweight/obesity, hyperglycemia and hyperlipidemia strongly contribute to increase the risk of these diseases (World Health Organization, 2013). In this sense, metabolic syndrome (MetS), a cluster of metabolic factors that include insulin resistance, impaired glucose tolerance, hypertension, dyslipidemia and central obesity, has emerged in the last decade as a serious public health challenge (Santhekadur, Kumar, Seneshaw, Mirshahi, & Sanyal, 2017), since it enhances the risk of type 2 diabetes mellitus and cardiovascular diseases in a greater extent than its individual components (Rodríguez-Monforte, Sánchez, Barrio, Costa, & Flores-Mateo, 2017).

The modification of diet is a keystone for preventing MetS development (Grosso et al., 2017). Providing consumers with a wider spectrum of healthy food options can offer new opportunities to modify the natural course of MetS. Agri-food sector is progressively addressing toward the development of plant-based products that contains, besides essential nutrients, bioactive ingredients endowed with disease-preventing effects. It has been well established that hypertension, abdominal obesity, inflammation and excessive activity of enzymes involved in glucose metabolism are associated to the development of MetS. Therefore, the production of multifunctional foods targeting these metabolic dysfunctions associated to MetS may represent an interesting approach in the primary and secondary prevention of this pathology.

Regular consumption of lentils (*Lens culinaris* L.), a key food of the Mediterranean diet, has been demonstrated to offer protection against metabolic alterations associated to MetS due to the presence of a wide range of bioactive compounds (Bautista-Expósito, Peñas, Dueñas, et al., 2018; Bautista-Expósito, Peñas, Silván, Frias, Martínez-Villaluenga, 2018; Martínez et al., 2016). Lentil is a rich source of phenolic compounds, mainly phenolic acids, catechin, procyanidins and flavonols that are able to scavenge free radicals or to activate pathways related to oxidative stress defense (Alshikh, de Camargo, & Shahidi, 2015), to inhibit the expression of pro-inflammatory molecules (Zhang et al., 2017), and to modulate the activity of enzymes involved in lipid and glucose metabolism (Zhang et al., 2015). Moreover, our group has

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recently identified biologically active peptides derived from legumin, vicilin and convicilin lentil proteins with antioxidant and angiotensin I-converting enzyme (ACE) inhibitory activity (Garcia-Mora, Frias, et al., 2015; Garcia-Mora, Peñas, Frias, Gomez, & Martinez-Villaluenga, 2015; García-Mora et al., 2017). Due to the presence of these compounds, together with its high content of resistant starch and dietary fiber, lentil represents a valuable ingredient for the elaboration of functional foods focused in amelioration of MetS disturbances.

Fermentation and enzymatic hydrolysis constitute promising processing technologies for development of lentil novel foods due to its ability to enhance their nutraceutical properties through increasing the content and bioaccesibility of bioactive compounds. Within this context, recent studies have shown that lentil and bean proteolysis by Savinase, a commercial food grade alkaline protease derived from *Bacillus spp*., released bioactive peptides and bound-phenolics with *in vitro* antioxidant and antihypertensive effects (Garcia-Mora, Peñas, Frias, & Martínez-Villaluenga, 2014; Garcia-Mora, Frias, et al., 2015; Garcia-Mora, Peñas, et al., 2015; García-Mora et al., 2017). This enzyme was most effective than other food-grade enzymes in releasing these bioactive compounds from legumes (Garcia-Mora et al., 2014). Additionally, there is evidence that lactic-acid fermentation with selected starter cultures enhanced the content of certain compounds with antioxidant, hypolipidaemic and ACE-inhibitory activities in lentil and other legumes (Jakubczyk, Karaś, Złotek, & Szymanowska, 2017; Kapravelou et al., 2015; Limón et al., 2015; Torino et al., 2013). However, the application of lactic acid fermentation for improving bioactive properties of legumes exhibits some limitations. Firstly, most lactic acid bacteria have low proteolytic activity that can difficult the release of bioactive peptides from legume proteins. Secondly, the reduction of pH during the course of lactic acid fermentation may decrease the solubility of legume proteins and phenolics, thus reducing their health-promoting properties. Combination of lactic-acid fermentation at selected pH with enzymatic hydrolysis by food-grade enzymes may overcome the limitations of fermentation technology increasing, at the same time, the health beneficial effects of legumes compared with the application of both technologies separately. A study recently performed by our group (Bautista-Expósito, Peñas, Dueñas, et al., 2018) has found the combination of both technologies at alkaline pH improved the health-promoting properties of lentil. However, studies at neutral or acid pHs that are more suitable for lactic-acid bacteria growth have not been performed so far. Lentil processed by combined fermentation and hydrolysis treatments could constitute valuable ingredients for designing novel functional foods such as dairy products, fruit juices or snacks.

Most of the studies focused on the evaluation of the impact of technological processes on biological effects of plant processed foods offer valuable information on the chemical identification of bioactive compounds responsible for their bioactivity. However, little is known on the stability and biological activity of constituents in vegetable processed foods subjected to gastrointestinal digestion. The use of *in vitro* gastrointestinal models are powerful alternatives to *in vivo* studies to evaluate the gastrointestinal stability of bioactive food compounds, since they have not ethical restrictions and are less expensive (Minekus et al., 2014).

The current study was, therefore, conducted to explore the feasibility of fermentation by *L. plantarum* CECT 748 in combination with enzymatic hydrolysis by an alkaline-serine protease (Savinase® 16 L) for producing a multifunctional ingredient aimed at relieving the metabolic disturbances associated to MetS. Fermentation and hydrolysis processes applied separately were also evaluated in order to elucidate their individual contribution when used in combination. The identification of peptides and phenolic compounds in the most bioactive fraction obtained after fractionation of lentil flour soluble fraction subjected to combined treatments and the influence of *in vitro* gastrointestinal digestion on the bioactive compounds and biological activity of processed lentil flour was also evaluated.

2. Materials and methods

2.1. Plant materials

Lentil seeds (*Lens culinaris* L. var. Castellana) were provided by Semillas Iglesias S.A. (Salamanca, Spain), ground in a coffee-mill (Moulinex, Allençon, France), passed through a 60-mesh sieve (0.5 mm pore size) and stored in a vacuum-sealed plastic bag at 4 °C until use.

2.2. Chemicals and reagents

Savinase® 16 L (16 KNPU/g) enzyme was purchase from Novozymes (Bagsvaerd, Denmark) and *L. plantarum* CECT 748 from the Spanish Type Culture Collection (CECT, Valencia, Spain). MidiTrap™ G10 gel filtration columns were from GE Healthcare (Barcelona, Spain). Angiotensin-I converting enzyme (ACE, EC 3.4.15.1), rat intestine α-glucosidase (EC 3.2.1.20), pepsin from porcine gastric mucosa (EC 3.4.23.1), pancreatin from porcine pancreas (EC 232-468-9) as well as other reagents were purchased from Sigma-Aldrich (Madrid, Spain) unless otherwise specified.

2.3. Lentil flour technological treatments.

Lentil flour was subjected to three different processing treatments:

(A) *Hydrolysis by Savinase (HS)*. Lentil flour was suspended in 1.5 L of sterile tap water (143 g/L final concentration), placed in 3 L Bioflo/Celligen 115 bioreactor (Eppendorf Iberica, Madrid, Spain) equilibrated at 37 °C and the pH was adjusted to 6.8 with 1 M NaOH. Then, Savinase was added (365 mg/L final concentration) and enzymatic hydrolysis was conducted under continuous stirring (300 rpm) at 37 °C, pH 6.8 for 25.5 h.

(B) *Fermentation using L. plantarum CECT 748 as starter culture (LP)*. Lentil flour fermentation was conducted in a 3 L Bioflo/Celligen 115 bioreactor (Eppendorf Iberica, Madrid, Spain) by mixing lentil flour suspended in tap water (143 g/L final concentration) with *L. plantarum* 748 from the Spanish Type Culture Collection (CECT) (2×10^8 CFU/L final cell density), previously grown in Man Rogosa Sharpe (MRS) broth (Pronadisa, Madrid, Spain) as recently described (Bautista-Expósito, Peñas, Dueñas, et al., 2018; Bautista-Expósito, Peñas, Silván, et al., 2018). Fermentation was performed under stirring (300 rpm) at 37 °C, pH 6.8 for 25.5 h.

(C) *Fermentation with L. plantarum CECT 748 combined with hydrolysis by Savinase (LPHS)*. Lentil flour suspension prepared in 1.5 L of tap water (143 g/L final concentration) was mixed with *L. plantarum* starter culture (2×10^8 CFU/L final cell density), previously propagated as described above, and with Savinase (365 mg/L final concentration). Combined treatments was performed in a 3 L Bioflo/Celligen 115 bioreactor (Eppendorf Iberica) under stirring (300 rpm) at 37 °C, *pH 6.8 for* 25.5 h.

Three independent experiments were carried out for each treatment. Lentil flour suspension without Savinase and *L. plantarum* addition was used as control. Soluble fractions from all samples were obtained by centrifugation (7500*g*, 5 °C, 15 min). Supernatants were heated at 70 °C during 10 min for enzymatic and bacterial inactivation. Finally, all samples were freeze-dried and stored under vacuum at −20 °C until further analysis.

2.4. Fractionation of LPHS by size exclusion chromatography

Soluble fraction obtained from LPHS sample was subjected to size exclusion chromatography using MidiTrap™ G10 columns containing Sephadex G-10. Briefly, freeze-dried sample was dissolved in distilled water (20 mg/mL) and filtered through 0.45 μm syringe filters. Column was equilibrated with 8 mL of deionized water. A sample volume of

0.3 mL followed by 0.3 mL of deionized water were added to the column allowing entering the packed bed completely and discarding the flow-through. Elution was carried out adding 2.5 mL of deionized water to the column. Five fractions (F1-F5) of 0.5 mL of eluate were collected, freeze-dried and stored at -20 °C until further analysis.

2.5. In vitro gastrointestinal digestion of LPHS

LPHS soluble fraction was subsequently subjected to a sequential *in vitro* digestion with porcine pepsin and pancreatin as previously described (Garcia-Mora et al., 2014). Briefly, LPHS was dissolved in simulated gastric fluid (SGF, 0.15 M NaCl, pH 2.5) and incubated at 37 °C for 15 min. Then 400 μL of a solution containing 0.59% (w/v) pepsin (3640 U/mg protein) in SGF was added and the pH was adjusted to 2.5 with 0.01 M HCl. Digestion was performed at 37 °C for 2 h in a Thermomixer™ orbital shaker (Eppendorf Iberica) with moderate agitation and subsequently stopped by raising the pH to 7.5 with 0.1 M NaOH. The pH of the digest obtained by gastric digestion was adjusted to 6.5 with 0.01 M HCl. Further, to simulate the duodenal digestion phase the following solutions were added: 150.8 μL of a bile salt mixture containing equimolar quantities (0.125 M) of sodium taurocholate and glycodeoxycholic acid, 46.08 μL of 1 M $CaCl_2$, 500 μL of 0.25 M Bis-Tris (pH 6.5), and 100 μL of pancreatin in SGF at pH 7.0. Digestion was conducted at 37 °C for 2 h and subsequently stopped by heating at 80 °C for 15 min.

2.6. Determination of peptide content

Peptide concentration was measured by Pierce Quantitative Colorimetric Peptide Assay kit (Fisher Scientific, Madrid, Spain) in permeates obtained by ultrafiltration through cellulose membranes of 10 kDa pore size (Millipore, Billerica, MA, USA). Results were expressed as mg of peptides/g of soluble fraction.

2.7. Determination of individual phenolic compounds

For the quantification of individual phenolic compounds, 2 g of lentil soluble fractions obtained from all samples were macerated in methanol:TFA:water 80:19.9:0.1 (v/v/v) at 4 °C for 16 h. Subsequently, they were centrifuged at 4000*g* and 5 °C for 20 min in a super-speed centrifuge (Sorval RC 5B). The extracts were concentrated at 30 °C under vacuum for methanol evaporation. For phenolic analysis, the dry extracts were dissolved in 10 mL of water. For purification, an aliquot (4 mL) was passed through a C18 Sep-Pak cartridge (Waters, Milford, MA, USA), previously activated with methanol followed by water.

Samples were analyzed using Hewlett–Packard 1100MS (Agilent Technologies, Palo Alto, CA, USA) chromatograph coupled to an HP Chem Station (rev.A.0504) data-processing software. Double online detection was carried out in the DAD using 280 nm and 370 nm as preferred wavelengths. Mass spectrometer (MS) connected to the HPLC system via the DAD cell outlet was used and detection was performed in an API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI source, triple quadrupole-ion trap mass analyser and controlled by the Analyst 5.1 software, following the method previously described by Barros et al. (2013).

Phenolic compounds were characterized according to their UV, mass spectra, retention times, and comparison with authentic standards when available. For quantitative analysis, calibration curves were prepared by injection of known concentrations of different standard compounds. The standards, vanillic, *trans-p*-coumaric acids, quercetin 3-*O*-rutinoside, quercetin 3-*O*-glucoside and kaempferol 3-*O*-rutinoside were obtained from Extrasynthese (Genay, France). Concentration of hexosides and quinic derivatives of protocatechuic, vanillic, caffeic, ferulic and *p*-coumaric acids were calculated by external calibration curves of their corresponding free acids while concentration of derivatives of quercetin and kaempferol were calculated by external calibration of kaempferol 3-*O*-glucoside and quercetin 3-*O*-glucoside. All samples were analyzed in duplicate. The content of total phenolic compounds was determined as the sum of the contents of individual phenolic compounds. Data were expressed as μg/g of soluble fraction.

2.8. Determination of total phenolic compounds in LPHS fractions

Total phenolic compounds of F1-F5 fractions obtained from LPHS sample by size exclusion chromatography were quantified by a colorimetric method using Folin-Ciocalteau reagent, as previously described (Bautista-Expósito, Peñas, Dueñas, et al., 2018; Bautista-Expósito, Peñas, Silván, et al., 2018). The absorbance was measured at 690 nm using a Synergy HT multi-well plate reader (BioTek, Winooski, VT, USA) and the results were expressed as mg gallic acid equivalents (GAE)/g of soluble fraction.

2.9. Identification of bioactive peptides by nanoUPLC-ESI-MS/MS

The proteomic analysis of the most active soluble fraction collected by size exclusion chromatography from LPHS was performed in the Proteomics and Genomics Facility (CIB-CSIC) by nanoUPLC-ESI-MS/MS, following the method previously described by García-Mora et al. (2017). Briefly, the most active fraction was purified using a C18 ZipTip reverse phase column (Millipore) and injected in the linear trap quadrupole Orbitrap Velos (Fisher Scientific). Peptides were separated in a AcclaimPepMap C18 column (inner diameter 75 μm, 15 cm long, 3 μm particle size) (Fisher Scientific) and eluted using a solvent gradient at a flow rate of 250 nL/min on a nanoEasy high-performance liquid chromatography coupled to a nanoelectrospray ion source (Fisher Scientific). The mobile phases used consisted of 0.1% formic acid/2% acetonitrile (solvent A) and 0.1% formic acid in acetonitrile (solvent B).

Proteome Discoverer 1.4.1.14 (Fisher Scientific) with MASCOT 2.3 was used to search the *Lens culinaris* database (NCBI 427 sequences). Database search parameters used were the following: the cysteine carbamidomethylation and methionine oxidation were established as fixed modifications; precursor ion tolerance, 10 ppm; fragment ion tolerance, 0.5 Da. Peptide sequences identification was validated through Percolator's algorithm using q-value ≤ 0.01.

2.10. Determination of ACE inhibitory activity

The ACE inhibitory activity of soluble fractions obtained from lentil processed by LP, HS and LPHS, in those sub-fractions obtained from LPHS sample (F1-F5) as well as in LPHS soluble fraction subjected to simulated gastrointestinal digestion (0.3 mg/mL final concentration) was determined according to a previously reported method (Garcia-Mora et al., 2014). Fluorescence was read every minute for 30 min at emission and excitation wavelengths of 335 and 405 nm, respectively, in a microplate reader Synergy HT (BioTek). The results are expressed as percent inhibition relative to the negative control having 100% enzymatic activity. All samples were analyzed in duplicate. IC_{50} values (concentration of sample in mg/mL that inhibits 50% of the ACE activity) were calculated plotting the non-linear regression sigmoidal dose-response curves in GraphPad Prism 4.00 (GraphPad Software Inc., San Diego, USA).

2.11. Determination of α-glucosidase inhibitory activity

The α-glucosidase inhibitory activity of lentil samples (1.7 mg/mL final concentration) was evaluated according to Vilcacundo, Martínez-Villaluenga, & Hernández-Ledesma (2017). Glucose concentration in the reaction mixtures was measured using the Amplex® Red glucose/glucose oxidase assay kit (Invitrogen, Carlsbad, CA, USA). Absorbance was measured using a Synergy HT plate reader (BioTek) at 560 nm. Glucose concentration was calculated using a linear standard curve (0–200 μM) from a freshly prepared 400 mM stock solution. All samples

were analyzed in duplicate. Percent inhibition of sucrase and maltase activity of intestinal α-glucosidase in the presence of lentil samples was calculated relative to the negative control having 100% enzyme activity.

2.12. Analysis of oxygen radical absorbance capacity (ORAC)

Radical scavenging activity of lentil samples was determined by fluorescence using the oxygen radical absorbance capacity (ORAC) assay previously reported (García-Mora et al., 2017). Fluorescence was measured in a Synergy HT microplate reader (BioTek) at emission and excitation wavelengths of 520 nm and 485 nm, respectively. A Trolox standard curve with a linear concentration range (0–160 μM) was prepared from a freshly made 1 mM stock solution. All samples were analyzed in duplicate. Results were expressed as mM of Trolox Equivalents (TE)/g of soluble fraction.

2.13. Determination of the potential protective activity of lentil samples against intracellular reactive oxygen species (ROS) production

Murine macrophage cell line RAW 264.7 (American Type Culture Collection, Rockville, MD, USA) was maintained in Dulbecco's modified Eagles's medium (DMEM) (Lonza, Madrid, Spain) supplemented with 10% fetal bovine serum (Lonza) and 1% penicillin/streptomycin (5000 U/mL, Lonza) at 37 °C in a humidified incubator containing 5% CO_2 and 95% air. Cell viability was determined using Cell titer 96 Aqueous One Solution assay kit (Promega, Madison, WI, USA). A non-toxic and physiological dose of 0.1 mg/mL was used to evaluate the potential protective activity of lentil samples. Intracellular ROS were measured by the dichlorofluorescein assay as previously reported (Martín, Cordero-Herrera, Bravo, Ramos, & Goya, 2014). Macrophages (2×10^5 cells/well) were pre-treated with lentil samples (0.1 mg/mL final concentration) dissolved in serum-free medium for 20 h. Cells were washed with PBS and incubated with 20 μM 2,7-dichlorofluorescin diacetate for 30 min. Then, cells were washed with PBS and treated with serum-free medium containing 2.5 mM *tert*-butyl hydroperoxide (t-BOOH). ROS production was monitored for 3 h by fluorescence at an excitation and emission wavelengths of 485 nm and 530 nm, respectively, using a Synergy HT plate reader (BioTek). Control cells with and without *t*-BOOH treatment were used as positive and negative controls, respectively. All samples were analyzed in duplicate. The results were expressed as percentage inhibition of ROS generation relative to *t*-BOOH-stressed cells.

2.14. Statistical analysis

Experimental data are expressed as the mean ± standard deviation of two replicates analyzed in duplicate. One-way analysis of variance (ANOVA) using the Duncan test was employed to compare the means. Differences were considered significant at $p < 0.05$. All statistical analyses were performed using Statgraphics Plus software, version 5.1. (Statistical Graphics Corp., Rockville, USA).

3. Results and discussion

3.1. Influence of processing on peptide, total and individual phenolic contents of lentil flour soluble fraction

The impact of hydrolysis with Savinase (HS), fermentation with *L. plantarum* (LP) and the combination of both treatments (LPHS) on the peptide concentration of lentil soluble fraction is shown in Table 1. All the processing technologies assayed led to a significant increase ($p < 0.05$) of peptides concentration compared to soluble fraction of unprocessed lentil (control sample). The extent of peptides release in LP was significantly lower ($p < 0.05$) than that observed for HS and LPHS, indicating that Savinase was the main responsible for liberation of peptides in LPHS. Previous studies performed by our group have demonstrated the higher ability of Savinase in releasing peptides from lentil proteins compared to other food grade enzymes (Garcia-Mora et al., 2014; Garcia-Mora, Frias, et al., 2015; Garcia-Mora, Peñas, et al., 2015). The lower production of lentil peptides by *L. plantarum* CECT 748 compared to the other treatments can be explained by the absence of the gene encoding the cell-wall bound proteinase (PrtP), involved in the hydrolysis of proteins to oligopeptides in the genome of this lactic acid bacterium (Liu, Bayjanov, Renckens, Nauta, & Siezen, 2010). However, *L. plantarum* produces peptide transporters and a wide range of intracellular peptidases that confer it the ability to grow and hydrolyse legume proteins, generating bioactive peptides, as it has been previously reported (Bautista-Expósito, Peñas, Dueñas, et al., 2018; Bautista-Expósito, Peñas, Silván, et al., 2018; Jakubczyk et al., 2017; Limón et al., 2015). Hence, it might be expected that the addition of Savinase, causing the hydrolysis of legume proteins to oligopeptides, would improve *L. plantarum* growth and release of peptides during lentil fermentation, as it has been observed in LPHS sample.

Hydrolysis, fermentation and the combination of both processing technologies have a noticeable influence on the phenolic content and composition of lentil soluble fraction (Table 1). Control lentil showed a concentration of total phenolic compounds, calculated as the sum of the contents of individual phenolic compounds, of 921 μg/g, concentration in the range of that recently reported for different lentil varieties (Bubelová, Sumczynski, & Salek, 2018). Flavonols, mainly kaempferol dirutinoside and flavan-3-ols such as (+)-catechin-3-*O*-hexoside and procyanidin dimer, were the major phenolic groups constituting more than 90% of total phenolic compounds, followed by hydroxycinnamic and hydroxybenzoic acids (6% of total phenolic compounds). This phenolic composition is similar to that previously found in different lentil cultivars (López et al., 2017; Zhang et al., 2017), where flavonoids were identified as the main extractable phenolic compounds.

After processing, the total phenolic content strongly decreased (31–35% reduction), regardless of the type of processing technology applied. No significant ($p > 0.05$) differences in the total phenolic concentration among HS, LP and LPHS samples were observed. The reduction of total phenolic content in processed lentil can be ascribed mainly to the decrease in flavan-3-ols concentration. Earlier studies have shown that the stability of flavan-3-ol monomers and dimers is pH dependent, and they are unstable and are degraded almost completely within few hours at pH > 6 (Albuquerque, Prieto, Barros, & Ferreira, 2017; Ho, Thoo, Young, & Siow, 2017; Li, Taylor, Ferruzzi, & Mauer, 2012; Zhu et al., 2002). The loss of flavan-3-ols during the course of hydrolysis, fermentation or combined treatments at pH = 6.8 may explain its lower content in processed lentil soluble fractions. Moreover, metabolic activity of *L. plantarum* may be contributing to the reduction of flavan-3-ols and phenolic acids in lentil subjected to fermentation or combined treatments. Savinase exhibits protease and esterase activities and this protease has been shown to cause the release of phenolic compounds linked to cell wall and other food matrix components in legumes at alkaline pH (Garcia-Mora, Frias, et al., 2015; Garcia-Mora, Peñas, et al., 2015; Bautista-Expósito, Peñas, Dueñas, et al., 2018). The reduction in the content of extractable phenolic compounds after Savinase hydrolysis can be explained by the lower pH used in the present work that is not the optimal pH for Savinase activity. Interestingly, HS, LP and LPHS exhibited higher content of *p*-hydroxybenzoic acid and flavonols in the soluble fraction than non-processed lentil that can be partially due to the low degradation of these compounds during processing. Moreover, the esterase activity of Savinase and the production of extracellular esterases by *L. plantarum* (Munoz et al., 2017) could be contributing to the increase of *p*-hydroxybenzoic acid and flavonols, results in consonance with the observations of Kwaw et al. (2018) and Garcia-Mora, Frias, et al. (2015), Garcia-Mora, Peñas, et al. (2015), who reported an increase of flavonol concentration after *L. plantarum* mulberry juice fermentation and lentil flour proteolysis with Savinase, respectively. Furthermore, the phenolic content and composition of LP

Table 1
Content of peptides (mg/g), total and individual phenolic compounds (μg/g) of soluble fraction obtained from control and processed lentil flour (HS, LP, LPHS).

Compounds	Lentil treatments			
	Control	HS	LP	LPHS
Peptides	55.65 ± 2.66[a]	192.72 ± 7.94[d]	68.80 ± 4.01[b]	180.19 ± 7.63[c]
Individual phenolic compounds				
Dimer Prodelphinidin (I)	75.31 ± 2.92[a]	nd	nd	t
Dimer Prodelphinidin (II)	5.60 ± 0.45[a]	43.15 ± 1.23[b]	t	t
(+)-catechin -3-O-hexoside	184.57 ± 10.33[c]	20.51 ± 0.25[b]	t	5.36 ± 0.19[a]
Dimer Procyanidin	108.06 ± 6.41[a]	nd	t	t
(+)-catechin	27.59 ± 3.14[b]	t	46.01 ± 1.27[c]	15.25 ± 2.30[a]
trans-p-coumaric acid derivative	9.83 ± 0.60[b]	1.77 ± 0.40[a]	t	t
trans-p-coumaric acid	37.23 ± 2.69[a]	nd	nd	nd
p-hydroxybenzoic acid	4.83 ± 0.20[a]	27.49 ± 0.99[b]	28.19 ± 0.99[b]	39.28 ± 3.12[c]
Vanillic acid	t	t	13.27 ± 0.49[a]	t
Kaempferol dirutinoside	371.97 ± 25.20[a]	453.98 ± 15.97[b]	429.74 ± 2.77[b]	456.83 ± 9.22[b]
Kaempferol rhamnoside-dihexoside	40.87 ± 1.53[a]	37.91 ± 2.99[a]	52.77 ± 0.79[b]	39.81 ± 1.62[a]
Isorhamnetin glucuronide	5.16 ± 0.81[a]	6.39 ± 0.45[ab]	7.75 ± 1.00[bc]	8.50 ± 1.01[c]
Kaempferol rhamnoside-dihexoside	14.28 ± 1.28[b]	4.75 ± 0.33[a]	12.09 ± 2.35[b]	12.99 ± 1.52[b]
Kaempferol hexoside-dirhamnoside (I)	27.12 ± 1.67[c]	10.38 ± 1.42[a]	nd	17.18 ± 0.59[b]
Kaempferol hexoside-dirhamnoside (II)	9.36 ± 0.15[a]	24.77 ± 1.76[b]	10.37 ± 2.37[a]	34.16 ± 0.18[c]
Total identified phenolic compounds	921.12 ± 42.91[b]	600.18 ± 3.42[a]	631.10 ± 41.20[a]	629.35 ± 19.94[a]

Data are the mean ± standard deviation of three replicates analyzed in duplicate. Different lowercase letters indicate statistical differences among treatments ($p < 0.05$. Duncan's test). HS: enzymatic hydrolysis with Savinase 16 L; LP: fermentation with *L. plantarum* CECT 748; LPHS: fermentation with *L. plantarum* CECT 748 combined with enzymatic hydrolysis with Savinase 16 L.; t: traces; nd: not detected.

and LPHS were rather similar, indicating that *L. plantarum* fermentation contributed to the phenolic profile of LPHS in higher extent than Savinase hydrolysis.

3.2. Influence of processing on biological activity of lentil flour soluble fraction

Table 2 summarizes the different biological activities evaluated in lentil flour processed by hydrolysis, fermentation or combination of both treatments.

ACE, an enzyme widespread in human body, plays a major role in regulating blood pressure (Nishimura, 2017). The search of food inhibitors of this enzyme as an effective approach for the management of arterial hypertension and cardiovascular diseases has been intensified in the last years.

Results of this study show that processing substantially enhanced the ACE inhibitory activity of lentil soluble fraction from 81% in non-processed lentil to 93–95% in treated lentil samples. No significant differences ($p > 0.05$) in ACE inhibition percentages between HS, LP and LPHS were observed. HS soluble fraction showed quite similar IC_{50} value than unprocessed lentil, while LP and LPHS exhibited significant ($p < 0.05$) lower IC_{50} values than HS. No significant differences ($p > 0.05$) in IC_{50} values between LP and LPHS samples were found, indicating that *L. plantarum* was the main contributor to the *in vitro* antihypertensive activity of LPHS sample. Bioactive peptides and phenolics, mainly flavonoids and phenolic acids, have been proposed as the main compounds involved in ACE inhibitory activity of lentils (García-Mora et al., 2017; Mamilla & Mishra, 2017). The increase in the content of flavonols and *p*-hydroxibenzoic acid after *L. plantarum* fermentation may partially explain the increase of ACE inhibitory activity found in LP and LPHS samples. In addition, *L. plantarum* has demonstrated the ability to produce ACE inhibitory peptides during fermentation of diverse legumes (Jakubczyk et al., 2017; Jakubczyk, Karaś, Baraniak, & Pietrzak, 2013). The formation of these peptides during lentil fermentation could be also involved in the ACE inhibitory activity of LP and LPHS found in the present work. As indicated before, several studies have shown that Savinase efficiently releases peptides with potent ACE inhibitory activity from legumes at alkaline pH. However, the pH used in the present work, lower than the optimal pH for the activity of this protease, could be limiting its activity and therefore, its contribution to potential antihypertensive activity of LP and LPHS samples might be reduced. In fact, lentil flour hydrolyzed by Savinase at pH = 8 or

Table 2
Bioactivity of soluble fraction obtained from control and processed lentil flour (HS, LP and LPHS), including: ACE-inhibitory activity expressed as percentage of inhibition relative to the control assay and IC_{50} in mg/mL. Intestinal α-glucosidase inhibitory activity expressed as percentage of inhibition of sucrase and maltase activities relative to the control assay. ORAC expressed in mM TE/g. Chemoprotective activity against oxidative stress expressed as percentage reduction of ROS generation relative to *t*-BOOH-stressed RAW 264.7 macrophages.

Bioactivity	Lentil treatments			
	Control	HS	LP	LPHS
ACE inhibition (%)	81.19 ± 6.23[a]	93.05 ± 1.82[b]	94.06 ± 0.66[b]	95.43 ± 2.14[b]
ACE inhibition (IC_{50}, mg/mL)	0.44 ± 0.04[b]	0.47 ± 0.01[b]	0.38 ± 0.00[a]	0.39 ± 0.00[a]
α-glucosidase inhibition (%)				
Sucrase activity	75.47 ± 4.56[d]	32.74 ± 0.20[b]	24.36 ± 1.77[a]	40.55 ± 2.50[c]
Maltase activity	nd	18.91 ± 1.22[a]	22.78 ± 3.40[ab]	25.03 ± 1.18[b]
ORAC (mM TE/g)	309.76 ± 23.32[b]	387.39 ± 24.09[c]	215.78 ± 12.31[a]	400.74 ± 35.35[c]
Inhibition of ROS generation (%)	nd	29.66 ± 2.15[b]	4.79 ± 2.84[a]	28.75 ± 0.33[b]

Data are the mean ± standard deviation of three replicates analyzed in duplicate. Different lower case letters indicate statistical differences among experimental groups ($p < 0.05$. Duncan's test). HS: enzymatic hydrolysis with Savinase 16 L; LP: fermentation with *L. plantarum* CECT 748; LPHS: fermentation with *L. plantarum* CECT 748 combined with enzymatic hydrolysis with Savinase 16 L; nd: not detected.

subjected to LPHS treatment at pH = 8.5 showed lower IC_{50} values (0.18 and 0.14 mg/mL, respectively) (Bautista-Expósito, Peñas, Dueñas, et al., 2018; Garcia-Mora et al., 2014) than those found in the present work for LP and LPHS samples, confirming the restricted role of Savinase in the ACE inhibitory activity in the present study.

Intestinal brush-border α-glucosidase enzymes, consisting in the complexes maltase-glucoamylase (MGAM) and sucrose-isomaltase (SI), are involved in the degradation of disaccharides into simpler sugars that are readily available for intestinal absorption (Simsek, Quezada-Calvillo, Nichols, & Hamaker, 2017). Hence, the inhibition of these enzymes by food compounds could be an economic strategy to control the post-prandial glycaemia, and consequently, to prevent type-2-diabetes.

Both α-glucosidase complexes consist of two catalytic subunits with different α-glucosidic catalytic properties or different substrates: MGAM has α-1,4-glucosidase activity and contributes to digestion of maltose and maltooligosaccharides, while SI with α-1,2, α-1,4, and α-1,6 activities can hydrolyze sucrose, maltose, isomaltose and maltooligosaccharides (Amiri & Naim, 2017). In the present work, we have evaluated the selective inhibitory potential of processed lentil samples for individual subunits of both α-glucosidase complexes, by using maltose and sucrose as substrates. Soluble fraction obtained from control lentil was able to selectively inhibit sucrase activity (75% inhibition), but inhibition of maltase activity was not observed (Table 2). Dietary phenolic compounds have been found to cause selective inhibition of intestinal α-glucosidases subunits (Simsek et al., 2017). Flavonols as kaempferol and quercetin glycosides have been suggested as the major contributors to the inhibitory activity of α-glucosidase in lentil (Zhang et al., 2015). In fact, flavonols are the main compounds in lentil soluble fraction (Table 1) that could explain its high inhibitory activity on α-glucosidase. Moreover, Simsek et al. (2017) found that (+)-catechin presented higher inhibition of one of the catalytic subunits of SI than those of MGAM, results in agreement with the selectively inhibition of sucrase activity by control lentil soluble fraction that contain this phenolic compound (27.5 μg/g).

Processing caused a significant reduction ($p < 0.05$) of α-glucosidase inhibition when sucrose was used as substrate (Table 2). The reduction of the content of some individuals phenolic compounds in lentil soluble fraction caused by processing such as (+) catechin and *p*-coumaric acid, which has been also described as potent α-glucosidase inhibitors (Pradeep & Sreerama, 2018), could explain the reduction in the sucrase inhibitory activity observed in HS, LP and LPHS samples. On the contrary, processing led to a strong increase of the inhibitory maltase activity of lentil soluble fraction, and LPHS showed the highest inhibitory potential for this enzyme. The enhancement of flavonols content during hydrolysis, fermentation and combination of both treatments could be related with the higher maltase inhibitory activity observed, since earlier studies have reported that flavonols exhibited specific inhibitory activity on duodenal maltase (Fontana Pereira et al., 2011; Şöhretoğlu et al., 2018). Among processed samples, LPHS presented the highest inhibitory potency against sucrase and maltase enzymes, suggesting that both *L. plantarum* fermentation and Savinase proteolysis contributed to the inhibition of α-glucosidase in this sample. Since Savinase caused significant higher release of peptides than *L. plantarum*, it can be hypothesized that some bioactive peptides produced by this protease are contributing to the inhibition of intestinal α-glucosidase in LPHS. This hypothesis is strengthened by the fact that several bioactive peptides formed during legume proteolysis has been found to inhibit this intestinal enzyme (Mojica, Luna-Vital, & González de Mejía, 2017). However, a possible contributing role of peptides released by *L. plantarum* in α-glucosidase inhibition by LP and LPHS samples cannot be ruled out since a previous study has shown the release of peptides with strong inhibitory activity against these enzymes from beans fermented with this lactic-acid species (Jakubczyk et al., 2017). In the view of the results obtained, it is evident that LPHS exhibited higher inhibitory potency than HS and LP against both α-glucosidases, suggesting that the combination of hydrolysis and fermentation could be a useful approach to obtain lentil ingredients aimed at the management of hyperglycemia. Moreover, HS was the most important contributor to the inhibitory effects of LPHS against SI, while the inhibitory activity of LPHS against MGAM can be mainly attributed to *L. plantarum*.

There is accumulative scientific evidence showing that oxidative stress, a state defined by elevated reactive oxygen species (ROS) levels, plays an important role in MetS development (Elnakish, Hassanain, Janssen, Angelos, & Khan, 2013). The antioxidant activity of processed lentil samples has been evaluated by ORAC assay and by the inhibition of ROS generation in RAW 264.7 macrophage cell cultures. ORAC assay measures the inhibition ability against the peroxyl radical-induced oxidation. As the peroxyl radical is the predominant initiating radical found in food systems, ORAC values are important indicators of food antioxidant activity (Xu, Jin, Peckrul, & Chen, 2018). Soluble fraction from non-processed lentil showed an antioxidant activity of 309.76 mM TE/g (Table 2), value in the line that those previously reported by extractable lentil fraction from different cultivars (Garcia-Mora, Frias, et al., 2015; Garcia-Mora, Peñas, et al., 2015; Padhi, Liu, Hernandez, Tsao, & Ramdath, 2017). This ORAC value could be linked to the abundant presence of (+)-catechin, trans *p*-coumaric acid, glycosides of flavonols or flavones, that have been suggested to be the main responsible for the antioxidant activity of pulses (Oomah, Caspar, Malcolmson, & Bellido, 2011; Zhang et al., 2015). However, the lack of a complete correlation between the content of total phenolic compounds and the antioxidant activity of pulses has been previously reported (Giusti, Caprioli, Ricciutelli, Vittori, & Sagratini, 2017; Zhang et al., 2015).

HS and LPHS showed significant ($p \leq 0.05$) higher ORAC values than non-processed lentil. On the contrary, LP exhibited lower antioxidant capacity than non-processed lentil. In a previous study, it has been demonstrated that Savinase released antioxidant peptides of low molecular weight (< 3 kDa) (Garcia-Mora, Peñas, Frias, & Martínez-Villaluenga, 2014). Since no significant differences in antioxidant activity between HS and LPHS were found, it can be suggested that antioxidant peptides produced by Savinase proteolysis from lentil soluble fraction were the most important contributors to the antioxidant activity of HS and LPHS samples. *L. plantarum* fermentation has been reported to increase the antioxidant activity of pulses either by increasing soluble phenolic compounds content or by releasing antioxidant peptides (Starzyńska-Janiszewska, Stodolak, & Mickowska, 2014; Torino et al., 2013). However, the different fermentation time and pH used in the present work could explain the reduction of antioxidant capacity of lentil soluble fraction after *L. plantarum* fermentation.

The protective role of soluble fractions obtained from non-processed and processed lentil against *t*-BOOH-induced oxidative damage was studied in RAW 264.7 macrophages. It was observed that the pretreatment of macrophage cultures with control lentil soluble fraction did not offer protection against oxidative damage stimulated by *t*-BOOH (Table 2). This finding does not match with the high chemical-based antioxidant activity determined by the ORAC assay, reflecting the fact that results from cellular and chemical analysis are not always completely correlated. Processing caused a notable increment of inhibition percentage of ROS production in stressed macrophages. HS and LPHS exerted significant ($p < 0.05$) higher protection against oxidative damage than LP, and no significant differences ($p > 0.05$) between both samples were observed, results that showed similar trend to that observed by the ORAC method. The hydrolysis of some complex phenolic compounds to simpler and biologically active compounds by *L. plantarum* (Rodríguez et al., 2009) could explain its greater inhibitory capability against ROS production compared to control lentil. Since HS, LP and LPHS showed similar content of soluble phenolic compounds and taking into account that the phenolic composition of LPHS sample was more similar to LP than HS (Table 1), the higher cytoprotective

Table 3
Recovery (%), content of peptides (mg/g) and total phenolic compounds (mg GAE/g), ACE-inhibitory activity (%), α-glucosidase inhibitory activity (%) and antioxidant activity measured by ORAC assay (mM TE/g) and as inhibition of ROS generation (%) in *t*-BOOH-stressed RAW 264.7 macrophages of F1-F5 fractions collected from LPHS by size exclusion chromatography.

	F1	F2	F3	F4	F5
Recovery (% of LPHS total weight)	12.76 ± 0.89	23.16 ± 1.72	35.15 ± 2.34	22.26 ± 2.34	6.67 ± 146
Peptides	759.66 ± 21.99^{e}	423.13 ± 13.57^{d}	75.20 ± 3.20^{a}	59.09 ± 2.00^{a}	267.83 ± 11.03^{c}
Phenolics	69.63 ± 0.96^{e}	44.23 ± 2.74^{d}	16.22 ± 0.16^{c}	6.55 ± 0.08^{a}	11.77 ± 0.04^{b}
ACE inhibition	96.00 ± 0.70^{b}	96.18 ± 0.25^{b}	nd	nd	nd
α-glucosidase inhibition					
Sucrase activity	nd	5.14 ± 2.34^{b}	4.45 ± 0.98^{b}	3.27 ± 1.75^{ab}	17.68 ± 0.20^{c}
Maltase activity	45.96 ± 1.09^{bc}	43.64 ± 1.23^{b}	44.87 ± 0.82^{bc}	46.69 ± 3.42^{bc}	49.01 ± 2.74^{c}
ORAC	575.50 ± 29.77^{e}	364.36 ± 29.16^{c}	104.21 ± 6.26^{a}	83.83 ± 6.68^{a}	325.75 ± 26.87^{b}
Inhibition of ROS generation	9.91 ± 1.27^{a}	15.08 ± 3.61^{ab}	27.36 ± 5.90^{c}	20.18 ± 2.64^{b}	27.68 ± 5.76^{c}

Data are the mean ± standard deviation of three replicates analyzed in duplicate. Different lowercase letters indicate statistical differences among fractions ($p < 0.05$. Duncan's test). LPHS: fermentation with *L. plantarum* CECT 748 combined with enzymatic hydrolysis with Savinase 16 L.; nd: not detected.

potential against oxidative damage of HS and LPHS cannot be attributed to phenolics. Keeping in mind that both samples exhibited similar peptide concentration and that Savinase is able to release peptides with peroxyl radical scavenging activity from lentil, it may be thought that antioxidant peptides released by Savinase hydrolysis provide the ability to HS and LPHS samples to inhibit ROS production in *t*-BOOH treated macrophages.

3.3. Fractionation of LPHS and characterization of the bioactive potential of the fractions

LPHS sample was fractionated in 5 fractions (F1-F5), and the content of peptides and phenolic compounds, as well as the biological activity of each fraction was studied. The results are summarized in Table 3. F1-F5 fractions represented 12.76%, 23.16%, 35.15%, 22.26% and 6.67% of the total weight of LPHS, respectively. As it can be observed, all fractions showed different content of bioactive compounds and biological activities. Only fractions F1 and F2 exerted inhibitory activity against ACE and F1 did not exhibit inhibitory potential against sucrase activity. However, all fractions showed inhibitory activity against maltase activity, high ORAC values and inhibition of ROS generation in RAW 264.7 macrophages. F1 was the fraction with the highest content of peptides and phenolics and antioxidant activity, measured by a chemical assay. Furthermore, F1 showed a notable MGAM and ROS inhibitory potential. Due to its higher biological activity, F1 was selected and their peptide and phenolic profile was analyzed by LC-MS/MS.

3.4. Characterization of LPHS-F1 peptide profile

Table 4 shows the most abundant peptides identified in the fraction F1 of LPHS sample, that corresponded to peptides with molecular masses within the range 838–1225 Da. These peptides derived from lentil storage proteins vicilin (allergen Len c 1.0102) and convicilin, and also from ADP-glucose pyrophosphorylase, a key regulatory enzyme involved in starch biosynthesis. These peptides contained some amino acid sequences that have been reported as ACE-inhibitors in BIOPEP database. It has been reported that ACE-inhibitory peptides are generally short chain peptides with 2–12 amino acids since crystallography studies have found that larger peptides cannot accommodate in the ACE active site (Iwaniak, Minkiewicz, & Darewicz, 2014; Natesh, Schwager, Sturrock, & Acharya, 2003). The C-terminal tripeptide strongly influences the ACE binding of inhibitors. It has been reported that peptides containing hydrophobic (aromatic or branched side-chains) in the C-terminal tripeptide are the most potent ACE inhibitors. Moreover, the presence of C-terminal Phe, Pro, Trp, Tyr, Leu, Ile, Val, and positively charged amino acids residues (Arg or Lys) and N-terminal hydrophobic amino acids with aliphatic chains such as Gly, Ile, Leu, and Val contribute notably to enhance the ACE inhibitory activity of peptides (Daskaya-Dikmen, Yucetepe, Karbancioglu-Guler, Daskaya, & Ozcelik, 2017; Gu & Wu, 2013; Liu, Fang, Min, Liu, & Li, 2018). Some of these structural features are present in the peptides identified in F1 fraction obtained from LPHS. In particular, these peptides showed the presence of Phe, Ile, Arg, Lys or Leu in the C-terminal tripeptide, supporting their potential ACE-inhibitory activity.

Table 4
Most abundant peptides identified in fraction F1 collected from LPHS by size exclusion chromatography.

Protein accessions	Sequence	Parent proteins	MH+ (Da)
29,539,111	SDQENPFIFK	Allergen Len c 1.0102	1224.59
164,512,510	HGDPEER	Convicilin	839.37
298,919,408	ATAFGLMK	ADP-glucose pyrophosphorylase small subunit S2 isoform	838.44

Recent studies have given important insight on the structural characteristics of antioxidant peptides, suggesting that the presence of Lys, Trp, Cys, and aromatic amino acids (Phe, His, Tyr and Trp) are important contributors of their radical scavenging capacities (Tian et al., 2015; Yang, Li, Lin, Zhang, & Chen, 2017). Furthermore, the presence of bulky hydrophobic amino acids at C-terminal region or polar/charged amino acids at the C1 position were related with peptide antioxidant activity determined by ORAC assay (Orsini Delgado et al., 2016). Hydrophobic amino acids with low isoelectric point (Ala, Gly, Val and Leu) at N-terminal region also provide high antioxidant activity to peptides (Li, Li, He, & Qian, 2011). Peptides identified in LPHS-F1 fraction contained charged amino acids in C1 position and some bulky amino acids in their sequence. In addition, peptide derived from ADP-glucose pyrophosphorylase exhibited Ala in N-terminus of the sequence. All these structural characteristics suggest the possible antioxidant role of the peptides identified in LPHS-F1. Moreover, they could potentially display α-glucosidase inhibitory activity since it has been reported that potent α-glucosidase inhibitory peptides identified up to now contain at least an amino acid with a hydroxyl group side chain (Ser, Thr, Tyr) and/or Pro, Lys, Arg and Met (Ibrahim, Bester, Neitz, & Gaspar, 2018; Zhang et al., 2016). The presence of some of these amino acids (Ser, Thr, Tyr, Lys, Arg) at the N-terminal position or Pro, Met, Ala at the C-terminal region confer highly α-glucosidase inhibitory activity (Ibrahim et al., 2018). The peptide derived from allergen Len c 1.0102 identified in LPHS-F1 fraction contained Ser at the N-terminal position and also Pro and Lys in its sequence. The other two peptides identified also contained Pro, Ala, Lys, Met or Arg, structural features of α-glucosidase inhibitors.

The size and structural features of the peptides contained in LPHS-F1 fraction allow us to postulate that all of them play an important role in the multifunctional activity of this sample.

Table 5
Phenolic compounds identified in fraction F1 collected from LPHS soluble fraction by size exclusion chromatography.

Compounds	Concentration (μg/g)
Dimer Prodelphinidin	0.133 ± 0.035
(+)-catechin-3-*O*-hexoside	0.095 ± 0.034
Kaempferol rutinoside-hexoside	0.510 ± 0.206
Isorhamnetin glucuronide	0.074 ± 0.019
Kaempferol rutinoside-rhamnoside (II)	t
Gallic derivative (I)	0.140 ± 0.033
Gallic derivative (II)	0.124 ± 0.027

Data are the mean ± standard deviation analyzed in triplicate. t: traces. LPHS: fermentation with *L. plantarum* CECT 748 combined with enzymatic hydrolysis with Savinase 16 L.

3.5. Characterization of LPHS-F1 phenolic profile

The phenolic content and composition of LPHS-F1 fraction is shown in Table 5. Flavonoids were the most abundant compounds, which accounted for 75% of the total phenolic content, being kaempferol rutinoside-hexoside the major compound detected. The other 25% corresponded to gallic acid derivatives. Flavonoids are potent scavengers of most of the oxidizing molecules, including singlet oxygen and other free radicals (Krishnan et al., 2018). Moreover, it has been reported that gallic acid shows a high inhibitory capacity of LDL-cholesterol oxidation and inhibition of peroxyl and hydroxyl radical induced supercoiled DNA strand (De Camargo, Regitano-D'Arce, Biasoto, & Shahidi, 2014). Zhang et al. (2015) found that kaempferol glycosides and procyanidin dimers play a significant role in the total antioxidant activity of hydrophilic extracts obtained from 20 different lentil cultivars. Furthermore, flavonoids and gallic acid are able to inhibit α-glucosidase activity (Miao et al., 2018; Šavikin et al., 2018; Wanget al., 2015). In this sense, kaempferol glycosides have been found to be one of the major contributors to α-glucosidase inhibition in lentil (Zhang et al., 2015), due to the ability of kaempferol to occupy the catalytic center of the enzyme, inducing conformational changes (Peng, Zhang, Liao, & Gong, 2016). The inhibitory effects of oligomeric procyanidins and kampferol on ACE have also been reported by several authors (Afonso, Passos, Coimbra, Silva, & Soares-da-Silva, 2013; Guerrero et al., 2012). According to all these findings of earlier studies, it can be postulated that the phenolic composition of LPHS-F1 plays a crucial role for its multifunctional activity.

3.6. Impact of simulated gastrointestinal digestion on bioaccesible peptides and phenolics and bioactivity of LPHS

In vitro gastrointestinal digestion is a useful approach to disclose changes in chemical composition, bioactivity and bioaccesibility of food compounds. Therefore, we evaluated the influence of simulated gastrointestinal digestion on the composition and bioactivity of LPHS sample (Table 6). As it can be observed, *in vitro* digestion enhanced more than 3-fold and 6-fold the peptide and phenolics contents, respectively. The content of peptides found in the digested sample is significantly higher than that found in LPHS lentil sample obtained at alkaline pH after gastrointestinal digestion (Bautista-Expósito, Peñas, Dueñas, et al., 2018), indicating that mild acidic conditions are more suitable than alkaline conditions for releasing peptides after digestive processes. These findings suggest that new peptides were released by the proteolytic activity of gastric and intestinal enzymes. In addition, as a result of the action of gastrointestinal enzymes, phenolic compounds bound to proteins could be released, increasing its bioaccesibility, as it has been previously reported (Pellegrini et al., 2017).

ACE inhibitory activity of LPHS sample increased significantly ($p \leq 0.05$) after simulated gastrointestinal digestion, as suggested by the lower IC_{50} value in the digested sample. This IC_{50} value is lower than that observed in a LPHS lentil sample obtained at alkaline pH after *in vitro* digestion (Bautista-Expósito, Peñas, Dueñas, et al., 2018). Our results indicate that new peptides with ACE-inhibitory activity may be liberated as consequence of the *in vitro* gastrointestinal digestion, improving the potential antihypertensive properties of LPHS. The increased ACE inhibitory activity observed could be also ascribed to extractable phenolic compounds released during digestive process. On the contrary, α-glucosidase inhibitory potency of LPHS for both sucrose and maltose substrates was significantly ($p \leq 0.05$) reduced by simulated gastrointestinal digestion. It can be inferred that some individual phenolic compounds that are able to inhibit these enzymes are degraded upon the effect of digestive enzymes and pH, as it has been recently reported in several vegetables (Burgos-Edwards, Jiménez-Aspee, Thomas-Valdés, Schmeda-Hirschmann, & Theoduloz, 2017; Orqueda et al., 2017). Similarly, α-glucosidase inhibitory peptides released by Savinase in LHPS could be hydrolysed during *in vitro* digestion, losing their inhibitory effects against this enzyme.

Table 6
Content of peptides (mg/g) and total phenolic compounds (mg GAE/g), ACE-inhibitory activity (IC_{50}, mg/mL), α-glucosidase inhibitory activity (%) and antioxidant activity measured by ORAC assay (mM TE/g) of LPHS soluble fraction before and after gastrointestinal digestion by size exclusion chromatography.

	Non-digested LPHS	Digested LPHS
Peptides	180.19 ± 7.63^a	653.58 ± 46.32^b
Phenolics	49.72 ± 4.20^a	299.10 ± 5.58^b
ACE inhibition	0.39 ± 0.00^b	0.19 ± 0.01^a
α-glucosidase inhibition		
Sucrase activity	40.55 ± 2.50^b	28.02 ± 0.92^a
Maltase activity	25.03 ± 1.18^b	nd
Antioxidant activity	400.74 ± 35.35^a	762.53 ± 36.00^b

Data are the mean ± standard deviation of three replicates analyzed in duplicate. Different lower case letters indicate statistical differences among treatments ($p < 0.05$. Duncan's test). LPHS: fermentation with *L. plantarum* CECT 748 combined with enzymatic hydrolysis with Savinase 16 L.; nd: not detected.

Digested LPHS sample exhibited an antioxidant activity almost 2-fold higher than undigested sample. These results agree with our previous findings showing that the gastrointestinal digestion of lentil protein hydrolysates obtained with Savinase released smaller peptide fragments and amino acids with higher antioxidant activity than the parent oligopeptides (Garcia-Mora et al., 2014; Garcia-Mora, Frias, et al., 2015; Garcia-Mora, Peñas, et al., 2015; García-Mora et al., 2017). Again, the contribution of free phenolic compounds released by the action of digestive enzymes in the increased antioxidant activity of digested LPHS cannot be ruled out. The antioxidant activity of digested LPHS observed in the present work is 37% higher than that found in digested LPHS lentil at alkaline pH (Bautista-Expósito, Peñas, Dueñas, et al., 2018; Bautista-Expósito, Peñas, Silván, et al., 2018).

The enhancement of most of the biological activities identified in LPHS after gastrointestinal digestion opens new opportunities for the application of lentils subjected to fermentation by lactic acid bacteria in combination with enzymatic hydrolysis by Savinase as a promising ingredient in the development of multifunctional foods. However, additional *in vivo* studies should be conducted in order to confirm the beneficial biological effects of this ingredient in metabolic disturbances associated to MetS.

4. Conclusions

Our approach, based on the combination of fermentation with *L. plantarum* CECT 748 and hydrolysis with Savinase 16 L (LPHS), efficiently improved the beneficial health effects of lentil regarding antioxidant, antihypertensive and hypoglycemic properties. These enhanced health-promoting properties of lentil can be attributed to the

action of both Savinase, responsible to the release of bioactive peptides and *L. plantarum* metabolic activity that caused the modification of phenolic compounds profile. Lentil flour subjected to combined hydrolysis-fermentation treatments was fractionated and the fraction with higher biological activity (F1) was selected and characterized. This fraction contained 3 peptides from allergen Len c1, convicilin and ADP-glucose pyrophosphorylase, as well as kaempferol rutinoside-hexoside, prodelfinidin dimer, and gallic acid derivatives as the most important bioactive compounds. This work gives also important insights about the effect of gastrointestinal digestion on the bioactivity of LPHS, confirming the enhancement of bioaccesible peptides and phenolic compounds with antioxidant and ACE inhibitory activities. This study contributes to elucidate the mechanisms of action underlying the demonstrated *in vitro* multifunctional effects related to MetS control of LPHS and provides the basis for further investigating the bioactivity of LPHS *in vivo*. The results could drive the agri-food sector to develop health-enhancing foods using LPHS as multifunctional health-promoting ingredient.

Declaration of interest

None.

Ethical statements

I have read and adhere to the publishing ethics of Journal of Functional Foods

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Conflict of interest

The authors declare no conflict of interest.

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